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Parasite-related modification of multiple traits in an isopod host

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ABSTRACT

Parasites are organisms that live on or in hosts, which they typically require to complete their life cycles. *Acanthocephalus dirus* is an acanthocephalan parasite that exhibits an indirect life cycle that requires two hosts, with a *Caecidotea intermedius* isopod as the intermediate host and fishes as the definitive hosts, in order to complete its life cycle. Infected *C. intermedius* individuals have been observed to have altered hiding behavior, in which they have an increased amount of time exposed to predators. Also, infected *C. intermedius* individuals have demonstrated an increase in activity patterns, which could increase their conspicuousness to visual predators. Infected *C. intermedius* individuals also exhibit pigmentation dystrophy, which makes infected isopods more conspicuous to visual predators than uninfected isopods.

Using a combination of field-based and laboratory-based behavioral trials, I examined the associations of *A. dirus* infection with behavior (hiding and activity) and body color of male and female isopods. I also examined the relationships between parasite characteristics (intensity and volume) and host trait modification. This study is the first to examine the relationships between effects of infection status in individual *C. intermedius* and multiple host traits, as well as the correlations among modified host traits and behaviors in both the field- and laboratory-based settings.

I tested for sex-associated effects on each of the traits using a Mann-Whitney U test. Spearman rank-order correlations were used to analyze the relationships between pairs of traits both in the field and laboratory experiments. To determine the potential impact of parasite characteristics (intensity and volume) on each of the traits, I used Spearman rank-order

correlation analysis on trait measures obtained in the field for behavior (hiding behavior and activity) and the laboratory for color (color score and % color).

Infected and uninfected isopods (males and females) differed in hiding behavior in the laboratory. Only infected and uninfected males differed in hiding behavior in the field. Infected and uninfected males did not differ in activity in the field and laboratory. Infected females crossed fewer grid lines than uninfected females in the field and laboratory. These changes in female activity appear to be more likely associated with pathological effects of infection than adaptive manipulation.

Infected isopods were lighter in color than uninfected isopods. The pigmentation dystrophy observed in this system may have been an adaptive mechanism used by parasites or a pathological side effect of infection. When using color score as the color measure, there was a positive correlation between color and activity that was present in only uninfected females in the field. However, there was not a correlation between these traits in the laboratory. The correlation between color and activity in uninfected females in the field may not be a meaningful correlation because it was expected that the correlation would occur in a different context, the laboratory, in addition to occurring in the field. Future research is needed to determine the potential significance of the correlations among traits identified in nature and disruptions to these correlations.

For the results obtained here, it appears that different traits may be modulated by different mechanisms because the correlations among traits were not consistent between different contexts.

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TABLE OF CONTENTS

TABLES

FIGURES

INTRODUCTION

A parasite is any organism that lives on or in a host in order to complete its development and reproduction (Moore 2002). Parasites are commonly found in multiple taxa and systems worldwide. As a consequence, most organisms will be infected by at least one parasite within their lifetime. However, parasite infection does not necessarily result in the death of a host. In some cases, parasites may modify the behavioral and physiological traits of one host (intermediate host) to increase the likelihood that transmission occurs to a second host (definitive host). For example, numerous studies have shown that parasites cause changes in intermediate host body color that increase conspicuousness to the predatory definitive hosts (Moore 2002; Kennedy 2006). Along with effects at the individual-level, parasites can have impacts at the population-, community-, and ecosystem-levels. They can affect growth and metabolism at the individual-level, reproductive rates at the population-level (Dobson 1988), predator-prey interactions at the community-level (Lafferty 1999), and energy flow and nutrient cycling at the ecosystem-level (Thomas et al. 1999). Due to the various and substantial impacts on other organisms, parasites have become a crucial point of interest when studying ecological interactions and mechanisms of evolution in the natural world.

Parasite life cycles can either be indirect or direct. Parasites that require one or more intermediate hosts have indirect life cycles. In contrast, the direct life cycle occurs when a parasite does not require an intermediate host (Moore 2002). Within each of these types of life cycle, parasites can either be actively or passively transmitted. Actively transmitted parasites typically search for their hosts, whereas passively transmitted parasites may be ingested by hosts along with their food or drink or carried to the host by a vector (*e.g.*, a blood-feeding

arthropod) (Moore 2002). Most protists, viruses, and bacteria use passive transmission (Murray et al. 1994; Moore 2002). *Giardia intestinalis* that causes 'backpacker's diarrhea,' is an example of a protist that has a direct life cycle with passive transmission. In this parasite, cysts exit the host through the feces, are transmitted to the next host through ingestion, encyst within the small intestine, and begin to divide (Moore 2002). In contrast, most parasitic arthropods actively seek their hosts to complete their life cycles (Moore 2002). An example of a parasitic arthropod is the isopod *Cymothoa exigua*, which takes the place of a tongue in a snapper (*Lutjanus guttatus*) (Brusca and Gilligan 1983). The snapper's tongue is reduced to a stub, allowing the isopod to take the space previously occupied by the tongue and possibly serve as a new tongue for the snapper (Brusca and Gilligan 1983).

In indirect life cycles, the parasites develop in one or more intermediate hosts and mature in the definitive host (Moore 2002). Parasite transmission to the definitive host can then occur when the intermediate host is consumed by the definitive host (Lafferty 1999). The significance of this predation event may have led to the selection favoring parasites that can induce behavioral, morphological, and physiological changes in intermediate hosts, which could increase parasite transmission to definitive hosts (Moore 2002; Thomas et al. 2005).

Uncinaria lucasi is an example of a nematode that has an indirect life cycle with passive transmission. This nematode lives within the lower small intestine of northern fur seal pups, *Callorhinus ursinus*, but intestinal infections do not occur in pups more than five months old (Moore 2002). This nematode does not have to leave the host because it uses transmammary transmission to move from mothers to offspring during lactation (Olsen and Lyons 1965). Once

the *U. lucasi* parasites are transmitted with milk when the pup nurses, the parasites can move directly to the intestine to reproduce, and the eggs are shed with the feces (Moore 2002). The eggs require most of the summer to emerge and develop within the rookery soil, and the larvae infect the seals that return the following year through tissue migration, which allows the larvae to distribute themselves in the blubber and mammary glands (Moore 2002).

Several crustaceans act as intermediate hosts and upon infection exhibit modified hiding behavior (Muzzall and Rabalais 1975c; Camp and Huizinga 1979; Hechtel et al. 1993; Bakker et al. 1997; Benesh et al. 2008), activity (Muzzall and Rabalais 1975c; Camp and Huizinga 1979; Pilecka-Rapacz 1986; Dezfuli et al. 1994; Beisel and Médoc 2010), body color (Bethel and Holmes 1977; Oetinger and Nickol 1981, 1982a, 1982b; Pilecka-Rapacz 1986; Bakker et al. 1997; Moore 2002), and attraction to disturbances or predators (Bethel and Holmes 1973; Hechtel et al. 1993; Lyndon 1996; Perrot-Minnot et al. 2007). Collectively, these changes can increase conspicuousness of the intermediate hosts and therefore increase the likelihood that predation occurs by definitive hosts (Camp and Huizinga 1979; Brattey 1983). However, not all changes in host phenotype are the result of parasitic manipulation. Changes could also be the result of pathological effects of parasite development, or be a host counter-adaptation to infection (Minchella 1985; Thomas et al. 2005).

Numerous studies have shown that infection of intermediate hosts is associated with modification of multiple traits. However, these studies have typically been completed in laboratory settings and have examined the traits independently of each other. Parasitic manipulation of host phenotype occurs along several dimensions, and the complex

relationships among these dimensions will determine the transmission benefits for the parasite (Poulin 2010). In other words, parasitic manipulation of host phenotype may affect multiple traits which could be correlated in terms of overall increased transmission to the definitive host. Furthermore, the manipulation of hosts by parasites can be pictured as a mosaic of multiple, altered traits. In order to define this mosaic, the traits themselves have to be quantified simultaneously (Thomas et al. 2005). Through this approach, the potential relationships between traits can be explored, which may aid in understanding the evolutionary forces behind a specific manipulation strategy (Benesh et al. 2008). Correlations between these host traits may be the true targets of manipulations, rather than the traits themselves (Coats et al. 2010). This idea relates to behavioral syndromes, or suites of correlated behavioral traits, which have become the distinguishing characteristic of animal personalities (Sih et al. 2004).

At the individual trait-level, two different altered traits may have a common developmental mechanism, which could result in positive correlations between the levels of each trait and lower costs of manipulation (Cézilly and Perrot-Minnot 2005). The alteration of additional traits can also synergistically increase the efficiency or specificity of the original manipulation through increasing the susceptibility to predation of an intermediate host by the definitive host while decreasing the susceptibility to predation by other predators unsuitable as definitive hosts for the parasite (Médoc et al. 2009). In contrast, altered traits could develop independently of each other, which would lead to uncorrelated trait levels. Parasites would then need to devote more energy in altering the traits, depending on the associated costs with the mechanism of each modified trait (Cézilly and Perrot-Minnot 2005). The respective impacts of the altered traits may also be dependent on external conditions, with one trait exhibiting a

major effect whereas the other trait exhibits no effect under certain conditions, and vice versa under different conditions (Poulin 2010).

Focusing on behavioral traits, the effects of parasites on behavior can often be differentiated into two main categories: 1) altered habitat preference, or 2) altered antipredator behavior. Altered habitat preferences often result in infected individuals spending more time in the open compared to uninfected individuals (Muzzall and Rabalais 1975c; Camp and Huizinga 1979; Hechtel et al. 1993; Bakker et al. 1997). Antipredator behavior in infected organisms (*i.e.,* hiding and evading) is usually reversed, along with changes in activity patterns (hyperactivity). For example, infected isopods were observed to be hyperactive and exhibit aberrant behaviors, such as moving towards the top of the substrate and wandering, as compared to less active and burrowing uninfected isopods (Muzzall and Rabalais 1975c; Camp and Huizinga 1979; Hechtel et al. 1993).

Acanthocephalans (Phylum: Acanthocephala), also known as both thorny-headed worms and spiny-headed worms, are trophically transmitted endoparasites (Kennedy 2006). These parasites require arthropods (isopods, amphipods, or insects) as intermediate hosts and vertebrates (*e.g.*, birds or fish) as definitive hosts (Kennedy 2006). Amphipods infected with the acanthocephalan parasite *Polymorphus paradoxus* skimmed on the surface or clung to floating objects in the water in response to either a direct or indirect disturbance, as compared to uninfected amphipods that would dive and burrow in response to disturbance (Bethel and Holmes 1973). Parasitized amphipods also display hyperactive behaviors as compared to unparasitized amphipods (Dezfuli et al. 1994, 2001). Infected isopods are attracted to fish

predators, as opposed to uninfected isopods that move away from fish (Hechtel et al. 1993). Parasitized amphipods also display an attraction to their predators, as compared to unparasitized amphipods (Baldauf et al. 2007; Kaldonski et al. 2007; Perrot-Minot et al. 2007).

The alteration of host behaviors by parasites can also be correlated with the intensity of parasites (Moore 2002). However, in nature it is difficult to demonstrate that the shift in behavior, even if correlated with intensity, is due to parasitism. For instance, organisms that live longer can grow larger (Benesh et al. 2009) and become exposed to more parasites over time (Baudoin 1975). Due to the greater intensity of parasites, they may increase activity levels and decrease antipredator behaviors, such as hiding and evading, because the energy that is used for those behaviors is being directed to the growth and survival of the parasites (Minchella 1985). The hosts may also show this change in behavior because they are older and larger, so their behavioral changes may be associated with senescence (Minchella 1985).

Acanthocephalans are exclusively parasitic with no free-living members (Kennedy 2006). They are characterized by their eversible proboscis that contains rows of recurved spines used in attaching to the intestine of their host (Kennedy 2006). Interspecific and intraspecific variation in the arrangement and number of the spines exists on a local (Brown 1987) and regional scale (Buckner and Nickol 1979). The spines are the only hard structure that acanthocephalans possess (Kennedy 2006). Because acanthocephalans have few organs useful in basing their taxonomy, they make classifying them on a systematic level difficult (Brown 1987). However, the distribution of acanthocephalans is widespread and contains species that

occur in terrestrial, marine, and freshwater environments, indicating that they are successful in their ability to infect hosts across all habitats of the world (Kennedy 2006).

Acanthocephala are divided into four classes: Archiacanthocephala, Eoacanthocephala, Palaeacanthocephala, and Polyacanthocephala, within which there are a total of 1,298 recognized species (Amin 2013). The terrestrial archiacanthocephalans are parasitic to birds, use terrestrial vertebrates as intermediate hosts, and are the earliest divergent lineage of acanthocephalans (Verweyen et al. 2011). The polyacanthocephalans are sister to the eoacanthocephalans, with the polyacanthocephalans being parasitic in fishes and crocodiles and the aquatic eoacanthocephalans being parasitic in fishes, amphibians, and reptiles (Verweyen et al. 2011). The palaeacanthocephalans demonstrate a paraphyletic assemblage, with final hosts being bony fishes, amphibians, and reptiles or birds and marine mammals (Verweyen et al. 2011).

The instances in which acanthocephalans occur in terrestrial hosts are closely related to diet (Kennedy 2006). For example, herbivores or carnivores with strict diets are not normally hosts to acanthocephalans, but species with omnivorous diets, such as freshwater fishes, are more likely to harbor acanthocephalans (Kennedy 2006). Furthermore, acanthocephalans are found to frequently parasitize birds that acquire them directly through feeding on arthropod intermediate hosts (Kennedy 2006). In aquatic hosts, marine fishes may be hosts to acanthocephalans, but patterns are difficult to detect in these fishes because of sampling problems or less extensive data sets (Kennedy 2006).

Three members of the paleacanthocephalan genus *Acanthocephalus* are found in North America, with *Acanthocephalus dirus* having the widest host and geographical range (Amin 1985). The other two species, *Acanthocephalus tahlequahensis* and *Acanthocephalus alabamensis*, are considered as southern and more local in distribution (Amin 1985). The three species are thought to be monophyletic, with *A. dirus* consistently shown as the ancestral species (Amin 1986). *Acanthocephalus dirus* has shown the greatest morphological variability of the three species, which is thought to be associated with the diversity of fishes that serve as definitive hosts (65 species from 16 families; Amin 1985). In comparison, *A. tahlequahensis* is found within four species and two families of fishes, whereas *A. alabamensis* is found within six species and four families of fishes (Amin 1986).

Acanthocephalans have a free-living egg stage which can either be released into the intestines of definitive hosts and dispersed with the feces or released from the body of the female after she is expelled from the host fish (Kopp et al. 2011; Wahl and Sparkes 2012). Transmission to the arthropod intermediate hosts occurs when leaf or algal material containing acanthocephalan eggs is consumed (Oetinger and Nickol 1982b). Inside the intermediate host, the parasite develops through the acanthor and acanthella stages and into the final infective cystacanth stage, which is capable of surviving transmission to the definitive host (Oetinger and Nickol 1982b). Acanthors are detected in the hindgut of intermediate hosts three days after the hosts are exposed to the eggs, early acanthellae are found 30 days after exposure, and cystacanths are observed between 80 and 90 days after exposure (Oetinger and Nickol 1982b).

The responses of intermediate hosts to acanthocephalan infection include changes in hiding behavior, activity, and mating behavior, as well as changes in appearance that increase their contrast against backgrounds (Camp and Huizinga 1979; Brattey 1983; Oetinger 1987; Weil 2002). All of these responses can increase conspicuousness to predatory definitive hosts, which is expected to favor transmission of the parasite to the definitive host (Camp and Huizinga 1979; Brattey 1983). For example, parasitized European isopods, *Asellus aquaticus,* are darker in color than uninfected individuals (Pilecka-Rapacz 1986). Darker color, as compared to a normally greyish protective color, may increase the chances of an infected isopod being eaten by a definitive host (Pilecka-Rapacz 1986). In contrast, the freshwater isopod *Caecidotea intermedius* in North America has a loss of pigmentation and looks lighter in color when infected with the cystacanth parasite *A. dirus* (Seidenberg 1973). Compared to a habitat dark in color that contains masses of vegetation and muddied grass roots, a light-colored infected isopod on the dark background has a greater chance of being detected by a potential definitive host than a dark isopod on the same background (Seidenberg 1973). This loss of pigmentation is due to the inability of the infected isopods to develop color and is more accurately described as pigmentation dystrophy (Oetinger and Nickol 1981).

Altered host behavior has been shown to lead to increased predation by definitive hosts (Hindsbo 1972; Bethel and Holmes 1977; Camp and Huizinga 1979; Brattey 1983; Bakker et al. 1997). *Acanthocephalus dirus*-infected *C. intermedius* individuals displayed shifts in two behavioral categories, habitat preference and antipredator behavior (Camp and Huizinga 1979). Laboratory experiments demonstrated that acanthocephalan-infected isopods that avoided hiding in any substrate and were hyperactive were more active than uninfected isopods that

burrowed into gravel or were under leaves (Camp and Huizinga 1979). These shifts in behavior could increase conspicuousness and consumption of the infected isopods by predatory definitive hosts.

I examined the host-parasite relationship between the acanthocephalan parasite species *A. dirus* (Van Cleave 1931) and its intermediate host, the isopod *C. intermedius*. Infection of *C. intermedius* individuals by *A. dirus* parasites occurs during the summer, and *A. dirus* parasites develop through the acanthor and acanthella stages into the cystacanth stage in approximately 90 days (Oetinger and Nickol 1982b). Cystacanths then prevail over infections occurring between September and May of the following year (Camp and Huizinga 1980). Numerous studies have examined acanthocephalan-isopod relationships, with a range of individual traits being affected across different species in North America and Europe (Camp and Huizinga 1979; Oetinger and Nickol 1981; Brattey 1983; Pilecka-Rapacz 1986; Hechtel et al. 1993; Dezfuli et al. 1994; Lyndon 1996; Sparkes et al. 2006; Benesh et al. 2008). These traits have been identified 30 separate times, with most studies being conducted in the laboratory setting (Table 1).

One of the traits that has been studied extensively is body color. Infected individuals exhibit pigmentation dystrophy (Oetinger and Nickol 1982b), which makes infected isopods more conspicuous to visually hunting predators than uninfected isopods (Camp and Huizinga 1979). In a similar host-parasite system within Europe, infected *A. aquaticus* individuals exhibit melanization (Table 2; Brattey 1983; Pilecka-Rapacz 1986; Dezfuli et al. 1994; Lyndon 1996;

Benesh et al. 2008). This may increase conspicuousness of *A. aquaticus* individuals to visual predators under these environmental conditions.

Infected *C. intermedius* individuals have also been observed to have altered antipredatory behavior, specifically altered hiding behavior, in which they have an increased amount of time exposed to predators (Table 1; Camp and Huizinga 1979; Hechtel et al. 1993). This is in contrast to uninfected *C. intermedius* individuals that remain hidden underneath rocks in stream ecosystems when exposed to predators. Similarly in Europe, the hiding behavior of infected *A. aquaticus* individuals was modified in relation to uninfected *A. aquaticus* individuals in ways that may increase consumption by predators (Table 2; Brattey 1983). Also, infected *C. intermedius* individuals have demonstrated an increase in activity patterns, which could also increase their conspicuousness to visual predators (Table 1; Muzzall and Rabalais 1975c; Camp and Huizinga 1979). This is contrary to uninfected *C. intermedius* individuals that decrease activity in the presence of predators. In Europe, infected *A. aquaticus* individuals have also been observed to have increased activity in comparison to uninfected *A. aquaticus* individuals (Table 2; Pilecka-Rapacz 1986). An increase in activity may increase exposure of infected isopods to visually hunting predators.

Using a combination of field-based and laboratory-based behavioral trials, I examined the associations of *A. dirus* infection with behavior (hiding and activity) and body color of male and female isopods. These trials were used to identify the relationships underlying parasite-related variation in phenotypic traits. I also examined the relationships between parasite characteristics (intensity and volume) and host trait modification. Parasite characteristics, such

as age, size, sex, and intensity, have the potential to impact host modification, with both positive and negative relationships being identified between the characteristic and the level of modification (Sparkes et al. 2004; Franceschi et al. 2008; Benesh et al. 2009; Dianne et al. 2012; Caddigan et al. 2014).

In some cases, competition among parasites sharing a host can also influence host modification if it limits the amount of resources available for parasites to allocate to manipulation (Dianne et al. 2012; Maure et al. 2013; Caddigan et al. 2014). This study is the first to examine the relationships between effects of infection status (infected or uninfected) in *C. intermedius* individuals and host traits, as well as among modified host traits and behaviors in both the field- and laboratory-based settings. Understanding the relationships among these modified host traits may provide insights into the evolution of both manipulation and transmission strategies in *A. dirus*. To examine these relationships, my study addressed the following questions:

1. Is *A. dirus* infection associated with the modification of focal traits in individual *C. intermedius* (hiding behavior, activity, and body color)? I hypothesized that infection would affect the normal state of the focal traits in individual *C. intermedius*. The focal trait expression of individual *C. intermedius* would be altered by *A. dirus* infection in ways that are expected to increase parasite transmission to the final host.
2. Is expression of the focal traits correlated in infected and uninfected *C. intermedius* individuals?

3. Is there repeatability in parasite-related expression of focal traits and the relationships among those traits, and is repeatability influenced by *A. dirus* infection in ways that could favor transmission?
4. Is the modification of focal traits in individual *C. intermedius* correlated with parasite characteristics (intensity and volume)? Positive and negative associations between parasite characteristics and trait expression have been identified in other host-parasite relationships (Sparkes et al. 2004; Franceschi et al. 2008; Benesh et al. 2009; Dianne et al. 2012; Caddigan et al. 2014).

METHODS

Study System

Caecidotea intermedius individuals were collected from Buffalo Creek, which is located 62 kilometers northwest of Chicago, Illinois (Fig. 1). In this stream, the macroinvertebrate community is dominated by *C. intermedius* individuals. This population exhibits a high infection rate of the trophically transmitted parasite *A. dirus* (prevalence = 54%, Sparkes et al. 2004). *Acanthocephalus dirus* exhibits an indirect life cycle that requires two hosts, with *C. intermedius* as the intermediate host and fishes as the definitive hosts, in order to complete its life cycle. At this site, the infection of *C. intermedius* individuals occurs during the summer (June-August) when isopods feed on detritus that contains parasite eggs (Oetinger and Nickol 1982b). During May, the adult isopods senesce, and the population becomes dominated by the next generation of *C. intermedius* individuals.

For *C. intermedius* individuals in Buffalo Creek there are two breeding seasons, which occur from March to May and from August to October (Sparkes et al. 2006). Isopods mating between March and May belong to the first cohort and are typically aged between 9 and 12 months (Mormann 2010). The fate of these isopods is to either be consumed by predators or senesce before the end of May. The other cohort consists of isopods that are the offspring of the first cohort and mate between August and October. Infected isopods that mate between March and May typically contain mature *A. dirus* (cystacanths) parasites, whereas infected isopods that mate between August and October contain a mixture of immature and mature *A. dirus* parasites (acanthor, acanthella, and cystacanth, Sparkes et al. 2006).

Parasite Infection and Multi-Trait Modification

To determine if *A. dirus* infection was associated with the modification of multiple traits (hiding behavior, activity, and body color), I examined each trait in the same individuals in two contexts (field and laboratory). To determine if *A. dirus* infection was associated with changes in hiding behavior of *C. intermedius* individuals, I used the percentage of time that individuals spent in the open. Time spent in the open was used instead of time spent hiding because it is the more relevant measure of predator exposure. To determine if *A. dirus* infection was associated with changes in activity of *C. intermedius* individuals, the number of grid lines that isopods crossed in individual experimental arenas was recorded as a measure of activity. To determine if *A. dirus* infection was associated with changes in body color of *C. intermedius* individuals, I analyzed digital images of individual isopods to obtain two measures of color (color type and percent of color present).

Consistency and Repeatability

Consistency describes variation that occurs within individuals in successive performances (Cummings and Mollaghan 2006). Repeatability describes the proportion of variance in a trait that occurs among individuals, rather than within individuals in a population (Boake 1989). It is used to determine the reliability of multiple measurements on the same individual (Lessells and Boag 1987), such as in the field and laboratory experiments involving behavior (hiding and activity). Repeatability uses intra-class correlation coefficients (Sokal and

Rohlf 1981) based on variance components that are derived from a one-way ANOVA (Lessells and Boag 1987). Repeatability, *r*, is calculated using the following equation: $r = \frac{{s_A}^2}{s^2} + {s_A}^2$, where ${s_A}^2$ is variance among individuals and s^2 is the variance within individuals over time (following Bell et al. 2009).

Field and Laboratory Experiments

Isopods were collected using hand-nets by running the net through vegetation, and by placing the net downstream while lifting rocks from the substrate. When rocks are lifted, the isopods are washed into the net by the current. A dichotomous color-scoring system was used in the field for distinguishing infected from uninfected isopods. Individual isopods were assessed as either appearing brown or yellow. Isopods that contained any yellow color on the dorsal surface were labeled as yellow and presumed to be infected, whereas isopods that contained a completely brown dorsal surface were labeled as brown and presumed to be uninfected. Sexual size dimorphism was used in the field for differentiating between male and female isopods. Adult male *C. intermedius* individuals are approximately 1.5 times larger than adult females (Keogh and Sparkes 2003). Male isopods are pear-shaped, whereas females are rectangular in shape (Keogh and Sparkes 2003). Using this approach, large, pear-shaped isopods were categorized as males, while small, rectangular isopods were categorized as females. Thus, there were four experimental groups (IM: infected males, UM: uninfected males, IF: infected females, and UF: uninfected females).

Field-based experimental trials were run over five non-consecutive days. On each of the days, I ran a total of 48 trials which included 12 replicates of each group (60 total replicates per group). In the stream, isopods occupy different microhabitats, and to account for this variation, I collected isopods from both microhabitats. Half of the replicates for each group was collected from an open area of the stream and the other half was collected from beneath rocks. For each field day, isopods were captured from the stream between 10:00 a.m. and 10:30 a.m. Hiding behavior and activity were then measured for each isopod. Trials were run in the field during April and May 2014 (April 11, April 18, April 26, May 3, and May 10).

Hiding Behavior (Field)

To determine if isopod hiding behavior was associated with parasite infection, I used a behavioral assay of hiding behavior. Individual isopods were randomly assigned to labeled holding arenas (small, round plastic containers, 7.8 cm diameter and 5.3 cm height filled with stream water) where they acclimated for approximately five minutes. Each isopod was then transferred to experimental arenas (rectangle plastic containers, 25.5 cm length x 14.5 cm width x 11.9 cm height, filled with stream water to an approximate height of 3 cm) within trays (88.6 cm length X 42.2 cm width X 15.6 cm height) (Fig. 2). Isopods were assigned to holding arenas based on their group classification, which was previously determined using a randomization chart (Lentner and Bishop 1986). This randomization ensured that isopods of each group were subjected to similar conditions. On the bottom surface, each experimental arena contained three flat rocks of different sizes (small, medium, or large). Small rocks were

2.8 cm length x 1.9 cm width x 0.9 cm depth; medium rocks were 3.1 cm length x 2.2 cm width x 1.1 cm depth; and large rocks were 3.5 cm length x 2.6 cm width x 1.2 cm depth. Each rock was elevated by three small pebbles that were glued to the rocks.

Hiding behavior trials were run between 10:30 a.m. and 2:30 p.m. at the field site. Observations of hiding behavior were made every five minutes for a total of 30 minutes, yielding six data points per trial (5, 10, 15, 20, 25, and 30 minutes). During each observation, I recorded whether the isopod was exposed (on top of the rock, on the side of the rock, or in the open) or in refuge (under the rock). For the analysis of hiding behavior for each isopod, I calculated the percentage of time that each isopod spent in the open by dividing the number of data points in which they were exposed by six.

Activity (Field)

To determine if the activity of isopods was correlated with parasite infection, grid sheets (2.5 cm x 2.5 cm grids) and experimental arenas (large, round plastic containers, 18 cm diameter and 8 cm height filled with stream water) were used (Fig. 3). Activity trials were run between 2:30 p.m. and 5:30 p.m. Following completion of the hiding behavior trials, isopods were transferred to the holding arenas prior to their use in the activity trials. The stream water was transferred from the rectangle experimental arena used for the hiding behavior trials into the round experimental arena used for the activity trials. Isopods were then transferred from the holding arenas to the round experimental arenas. Observations for each isopod lasted five

minutes. During each observation, the number of grid lines that the isopod crossed was recorded using a tally counter.

Individual isopods were placed into individually labelled 50 mL polyethylene bottles filled with stream water at the end of the field-based trials. The isopods were held in these stream-filled bottles for approximately one hour while being transported to the DePaul laboratory. Once in the laboratory, the isopods were transferred to individual holding arenas filled with stream water and left to acclimate overnight. Six 5-gal (18.9 L) coolers were used to house stream water in the laboratory overnight and between days. These coolers were aerated by Whisper 100 air pumps.

Hiding Behavior and Activity (Laboratory)

Hiding behavior and activity were recorded in the laboratory following the same approach as the field-based experiment (see above). Laboratory trials were run the day after the field trials during the same time period. At the end of the laboratory-based trials, each isopod was placed into an Eppendorf tube and frozen until sex determination occurred. Freezing the isopods retains the body color present in the field (Hargeby et al. 2004). Each isopod was then sexed, using anatomical characters (presence of oöstegites or a brood pouch in females, presence of hemi-peni in males, Weil 2002), prior to their use in the color analysis.

Body Color

To determine if *C. intermedius* individual body color was associated with *A. dirus* infection, I used image analysis software (ImageJ) on digital images captured for each isopod. Digital images were captured using a Cannon PowerShot SX40 HS digital camera (scene mode: close up, focal length: 4 mm, aperture: F4, shutter speed: 1/40, sensitivity: ISO100, image size: 4000 x 3000 pixels, image quality: fine, focus mode: auto) in the DePaul University greenhouse located on the Lincoln Park campus. I constructed a tray (21 cm length x 13.6 cm width) to take pictures of each isopod.

The isopods were distinguished by their individual identification codes. Within each code, BC identified Buffalo Creek as the study site, the first number identified the field day, and the second number identified the individual isopod number. One-centimeter sections of a ruler were cut and placed with the isopods, along with a section of a stimulus array modified from Lenneberg and Roberts (1956). The section of the stimulus array included columns 5.5 YR through 2.5 Y, where YR means yellow-red and Y means yellow (Fig. 4). The ruler and stimulus array were placed in a consistent location on the tray for each picture (Fig. 5). The color strip ranged from white to brown and included the range of colors present in infected and uninfected isopods. The one-centimeter sections of a ruler were used as a reference for size. I defrosted and laid flat one isopod immediately before taking a picture and kept the rest of the isopods on ice to preserve color and avoid degradation. I took at least two pictures of the dorsal side of each isopod and used the image that provided the highest resolution for analysis. Images of the dorsal side were used because isopods are most likely observed by predators in

this orientation (Benesh et al. 2008). Pictures were taken between 8 a.m. and 11 a.m. each day and at a consistent height (12.7 cm).

I evaluated the body color of each individual isopod based on a score that ranged from 1-9, with 1 being completely absent of pigmentation (colorless) and 9 containing full pigmentation (dark brown) (Fig. 5). I also measured the percentage of dark brown color present (% color) for the entire area of each isopod by comparing the areas of dark color to the total area of the isopod using ImageJ analysis software (imagej.nih.gov/ij/). I recorded the body size of each isopod from ImageJ by measuring the surface area of a two-dimensional image. In lightly pigmented isopods, the intestine was usually visible, and this was excluded in order to avoid any effects that ingested food might have on the analysis (Hargeby et al. 2004).

After the analysis of body color was completed, I dissected the isopods and determined infection status. For each parasite recovered, I recorded the developmental stage, length, and width of each *A. dirus* parasite. Length and width were used to calculate parasite volume [((π x length x width^2)/6) following Dezfuli et al. 2001]. I also recorded parasite intensity (number per infected isopod, following Bush et al. 1997). The developmental stage of each parasite was identified as either acanthella or cystacanth using three measures of development (following Hasu et al. 2007). A parasite was categorized as a cystacanth if the reproductive structures were developed (ovaries for females or testes for males), invagination of the proboscis had occurred, and the spines located on the proboscis were fully developed into hooks. A parasite lacking any of these developmental measures was recorded as an acanthella.

Statistical Analysis:

To determine if body size should be accounted for in the analysis of body color and behavior, I used correlation analysis (Spearman rank-order correlation due to non-normal data despite transformation). In most cases, there was no correlation between body size and the trait, and as a consequence, body color and behavior were not size-adjusted for analysis. The relationship between parasite infection and expression of body color and behavior were analyzed separately for males and females because sex-specific effects were identified here and have been identified previously in this host-parasite relationship (e.g., mating behavior, Sparkes et al. 2006). The values used were generally not normally distributed (Shapiro-Wilk test) despite transformations; therefore, non-parametric tests were used for the analysis. I also examined whether body size was affected by infection status and sex using a two-way ANOVA (fixed effect model, Systat 11).

To determine the level of consistency and repeatability of each behavior (hiding and activity) measured in the field, I used two approaches. For consistency, I used a Spearman rank-order correlation analysis to determine if measures in the field were correlated with measures in the laboratory. For repeatability, I used intra-class correlation coefficients to determine if multiple measurements on the same individual were reliable in the field and laboratory for behavioral traits.

Spearman rank-order correlations were used to analyze the relationships between pairs of traits for each of the four groups (infected males, uninfected males, infected females, and uninfected females) both in the field and laboratory experiments. Partial correlation

coefficients were used to account for confounding effects of additional variables (Conover 1980; Zar 2010). Pairs of variables included comparisons of color score and hiding behavior, color score and activity, % color and hiding behavior, % color and activity, and hiding behavior and activity.

To determine the potential impact of parasite characteristics (intensity, average volume, and total volume) on each of the traits, I used Spearman rank-order correlation analysis on trait measures obtained in the field for behavior (hiding behavior and activity) and the laboratory for color (color score and % color).

RESULTS

To determine the relationships among isopod sex, infection status, and behavior, I ran 240 trials over five non-consecutive days. The field-trials were repeated in the laboratory setting using the same experimental set-up to determine if there was consistency and repeatability in trait expression in different contexts. The assumption was that the uninfected isopods in the field-trials exhibited the normal behavioral state. In the field, I used body color to tentatively assign infection status (infected and uninfected) and body size to assign isopod sex. On completion of the dissections, I was able to correctly assign infection status and sex of the isopods. In terms of infection status, 89% of the isopods were classified correctly. In terms of sex, 100% of the isopods were classified correctly. When appropriate, isopods were reassigned to the correct groups for analysis. Several trials were removed from the analysis because of isopod mortality or because an error was made during data collection in the laboratory experiment. The analysis was run on a total of 179 isopods (49 infected males, 40 uninfected males, 48 infected females, and 42 uninfected females), which represents 75% of the original isopods collected. The loss of 25% of the trials is a concern that needs to be addressed in future studies. Of the total number of isopods tested, 45% of infected isopods and 45% of uninfected isopods were collected from the open (exposed) microhabitat in the study system.

Parasite Infection and Multi-trait Modification

Body Size

To examine the effects of sex and infection status on body size, I ran a two-way analysis of variance. The interaction term between infection status and sex was not significant ($F_{1,174}$=0.6, p=0.4), and there was no effect of trial date on body size ($F_{1,174}$=1.3, p=0.3). There was an effect of sex ($F_{1,174}$=56.4, p<0.001) and infection status ($F_{1,174}$=15.1, p<0.001, Fig. 6). Male isopods were larger than females, and infected isopods were larger than uninfected isopods.

Hiding Behavior

The relationships among isopod sex, infection status, and hiding behavior are summarized in Table 3. I used a Mann-Whitney U-test to determine if hiding behavior differed between infected and uninfected isopods and between sexes. Infected and uninfected isopods differed in time spent out in the open in the field ($U_{97,82}$=4679, p=0.04) and in the laboratory ($U_{97,82}$=5207, p<0.001). Males and females did not differ in hiding behavior in the field ($U_{89,90}$=3853, p=0.66) or in the laboratory ($U_{89,90}$=3664, p=0.23). Within the sexes, infected males spent more time out in the open than uninfected males in the field ($U_{49,40}$=1228, p=0.04) and in the laboratory ($U_{49,40}$=1228, p=0.01). Infected and uninfected females did not differ in hiding behavior in the field ($U_{48,42}$=1099, p=0.45) but did differ in hiding behavior in the laboratory ($U_{48,42}$=1370, p=0.001).

Activity

The relationships among isopod sex, infection status, and activity are summarized in Table 3. To determine if activity differed between infected and uninfected isopods and between sexes, I used a Mann Whitney U-test. Infected and uninfected isopods differed in the number of grid lines crossed in the field ($U_{97,82}$=3191, p=0.02) but did not differ in the number of grid lines crossed in the laboratory ($U_{97,82}$=3817, p=0.64). Males and females did not differ in activity in the field ($U_{89,90}$=4216, p=0.54) or in the laboratory ($U_{89,90}$=4167, p=0.64). Within the sexes, infected and uninfected males did not differ in activity in the field ($U_{49,40}$=916, p=0.60) or in the laboratory ($U_{49,40}$=924, p=0.65). Infected females crossed fewer grid lines than uninfected females in the field ($U_{48,42}$=667, p=0.01) but not in the laboratory ($U_{48,42}$=982, p=0.83).

Consistency and Repeatability of Behavior

The relationships among isopod sex, infection status, and consistency and repeatability of the field and laboratory behavior (hiding and activity) are summarized in Table 4. Activity measures were generally both consistent and repeatable between the field and laboratory. Measures of hiding behavior showed more variation in consistency and repeatability. Specifically, in two out of the four groups, there was consistency, but not repeatability. Repeatability estimates that are less than zero occur when large standard error bars surround estimates of between-individual variance components (Bell et al. 2009).

Body Color

Two measures of color were used for the analysis (Fig. 7). Infected isopods were generally light-colored (color score range=1-3), with most of the body lacking pigmentation (% color=1-6%). In contrast, uninfected isopods were generally dark brown in color (color score range=7-9), with most of the body containing pigmentation (% color=80-100%). I used a Spearman rank-order correlation to examine the relationship between color score and % color for each of the four groups (infected males, uninfected males, infected females, and uninfected females). The color score and % color were correlated for both infected isopods (males: r_s=0.69, p<0.001; females: r_s=0.68, p<0.001) and uninfected male isopods (r_s=0.55, p<0.001), but not for uninfected female isopods (r_s=0.28, p>0.05). I ran analysis on each variable separately because each represents a different measure of color.

The relationships among isopod sex, infection status, and body color are summarized in Table 3. I used a Mann-Whitney U-test to determine if color score and % color differed between infected and uninfected isopods and between sexes. Infected and uninfected isopods differed in color score ($U_{97,82}$=948, p<0.001) and % color ($U_{97,82}$=724, p<0.001). Males and females did not differ in color score ($U_{89,90}$=4016, p=0.98) or % color ($U_{89,90}$=4129, p=0.70). Within sexes, infected males and females had lower color scores than uninfected males and females (males: $U_{49,40}$=233, p<0.001; females: $U_{48,42}$=245, p<0.001). Infected males and females also had lower % color values than uninfected males and females (males: $U_{49,40}$=205, p<0.001; females: $U_{48,42}$=164, p<0.001). Overall, infected isopods had lighter body coloration and less brown pigmentation than uninfected isopods.

Multi-Trait Correlations

I used Spearman rank-order correlations to explore the relationships between pairs of traits in both the field and in the laboratory for each of the four groups. To examine the relationships among the traits (hiding behavior, activity, and body color), I used partial correlation analysis to account for confounding effects of each additional variable (Conover 1980; Zar 2010; Table 5). When using color score as a color measure, there was a positive correlation between color and activity that was present in uninfected females only in the field (Fig. 8). This indicates that females that were more dark brown in color were also more active. However, there was no correlation between color score and activity in uninfected females in the laboratory (Table 5). There were no signification correlations between hiding behavior and either activity or body color for uninfected isopods in the field (Figs. 8, 9). There were also no significant correlations between % color and activity for uninfected isopods in the field or in the laboratory (Fig. 9). In infected isopods, color score and % color were not correlated with activity in males or females in the field or in the laboratory. Similar to uninfected isopods, there were no signification correlations between hiding behavior and either activity or body color for infected isopods in the field (Figs. 8, 9).

We identified only one trait combination that was correlated in the field (Table 5; Figs. 8, 9). Color score and activity were positively correlated in uninfected females. This correlation was not present when the uninfected females were examined in the laboratory the following day after they were examined in the field. One other correlation emerged in the laboratory that was present in uninfected females but not uninfected males, infected males, or infected

females. Activity and hiding behavior were positively correlated for uninfected females in the laboratory.

Parasite Characteristics and Multi-Trait Expression

I used Spearman rank-order correlations to explore the potential impact of parasite characteristics (intensity, average volume, and total volume) on trait measures obtained in the field for behavior (hiding behavior and activity) and the laboratory for color (color score and % color) in infected male and female isopods. The relationships between parasite characteristics and host trait expression are summarized in Table 6. There was a positive relationship between body size of male isopods and both average volume of parasites and total volume of parasites. There were no other significant correlations between parasite characteristics and traits of the male and female isopods. Overall, larger male isopods contained a greater parasite volume (average and total).

The relationships among host sex, host body size, and parasite characteristics are summarized in Table 7. There was no detectable effect of host sex on parasite intensity, average parasite volume, or total parasite volume.

DISCUSSION

I examined the relationships of *A. dirus* infection with behavior (hiding and activity) and body color of male and female isopods to identify the correlations underlying parasite-related variation in phenotypic traits. I also examined the relationships between parasite characteristics (intensity and volume) and host trait modification. This study was the first to examine the correlations between infection in *C. intermedius* individuals and associated effects on host traits, as well as among modified host traits in both the field- and laboratory-based settings. Understanding the relationships among these modified host traits could provide insights into the evolution of both host manipulation and transmission strategies in *A. dirus*. Studying the manipulation and transmission strategies of *A. dirus* may also provide insights into the manipulation and transmission strategies of other parasites. This could be of interest to parasitologists and people with a non-biological background alike because any organism (including humans) could be infected by a parasite at any point in their lifetime. To examine the relationships among the modified host traits, the study addressed several questions, which are discussed in detail below.

Modification of Focal Traits: Hiding Behavior, Activity, and Body Color

Previous studies on hiding behavior have shown that infected *C. intermedius* individuals displayed altered hiding behavior when observed individually (Muzzall and Rabalais 1975c; Camp and Huizinga 1979; Hechtel et al. 1993). Also, previous studies have revealed that infected *C. intermedius* individuals have altered activity patterns when assessed individually

(Muzzall and Rabalais 1975c; Camp and Huizinga 1979). Previous studies on body color have also shown that pigmentation dystrophy is expected to increase the visibility of infected isopods, as opposed to uninfected isopods, to visual predators when examined individually (Camp and Huizinga 1979; Oetinger and Nickol 1981; Hechtel et al. 1993; Weil 2002).

The first part of this study examined if *A. dirus* infection would affect the normal state of hiding behavior in individual *C. intermedius*. The prediction was that infection would alter hiding behavior in *C. intermedius* individuals in ways that would increase parasite transmission to the final host. The results obtained here showed that there was a difference in hiding behavior between infected and uninfected males and females in the laboratory, and only a difference in hiding behavior between infected and uninfected males in the field. The hypothesis and prediction were therefore supported in the laboratory for males and females and partially supported for males in the field. If this study were to be repeated, increasing the sample size may have eliminated the inconsistency among the results. Increasing the sample size would improve estimates of the repeatability of hiding behavior (Bell et al. 2009).

For the second hypothesis, I examined if *A. dirus* infection influenced the normal state of activity in individual *C. intermedius*. The prediction was that infection would alter activity in *C. intermedius* individuals in ways that would increase parasite transmission to the final host. A previous study showed that *A. dirus* infection was associated with an increase in activity (Muzzall and Rabalais 1975c; Camp and Huizinga 1979). The results observed here showed that there was only a difference in activity between infected and uninfected females, which

occurred only in the field. In the field, infected females had lower activity levels than uninfected females. Thus, the original hypothesis and prediction were not supported.

Because only females were affected, the change may not be associated with parasite transmission because this type of effect should occur in both females and males. A pathological effect of infection may be a more likely explanation for this pattern because females appear to be more prone to pathological effects than males in acanthocephalan-host relationships. For example, the reproductive organs of females undergo atrophy during infection, whereas the reproductive organs of males are unaffected (Oetinger 1987; Sparkes et al. 2006; Bierbower and Sparkes 2007; Dezfuli et al. 2008).

It has been proposed that parasites may redirect energy allocated to ovarian development and egg production to somatic growth (Oetinger 1987), which may benefit the parasite by providing more space for growth (Baudoin 1975). Decreased energy expenditure towards host reproductive effort could also reduce host mortality and increase host viability by decreasing the energy and risks associated with reproductive effort (Baudoin 1975). This would also be beneficial for the parasite because this may increase its probability of surviving to a reproductive age and its number of reproductions overall (Baudoin 1975). This type of effect may be more pronounced in females because they are smaller than males, and as a result, the parasites may have to induce more changes in the females than males to complete their own development.

Increased energy allocated to host growth may also aid the parasite if there is a positive correlation between host size and parasite fecundity (Baudoin 1975). More energy could be

available to the parasite due to benefits that arise from a larger host being a better competitor. Because there is no difference in the reproductive effort between infected and uninfected male isopods, less energy may be allocated to non-reproductive efforts such as somatic growth and activity.

The last part of this study examined if there was modification of isopod body color associated with parasite infection. The hypothesis was that *A. dirus* infection would affect the normal state of body color in individual *C. intermedius*. The prediction was that body color of individual *C. intermedius* would be altered by *A. dirus* in ways that are expected to increase parasite transmission to the final host. The results showed that there was an effect of *A. dirus* infection on color score and % color for female and male isopods. Infected isopods were less dark brown in color and contained less total color pigment than uninfected isopods. Therefore, the hypothesis and prediction were supported. These lighter colored isopods contrast with a habitat that is dark brown, dark green, or black in color, which could increase chances of *A. dirus* parasites being transmitted to a visual predatory final host (Seidenberg 1973; Camp and Huizinga 1979; Camp and Huizinga 1980; Oetinger and Nickol 1982a). The pigmentation dystrophy has been proposed to be due to the parasite-related interference with protein metabolism, possibly disrupting the normal development of pigmentation (Oetinger and Nickol 1982b). Thus, pigmentation dystrophy could be a pathological side-effect of infection (due to a need for amino acids in the developing parasite) or the direct result of parasite manipulation (parasites prevent the isopods from using the amino acids to produce color). Another possibility is that the color change was a pathological side-effect of infection that was then favored by selection because it increased conspicuousness of infected hosts to definitive hosts.

In summary, for hiding behavior, there was a difference in hiding behavior between infected and uninfected isopods in the laboratory and only a difference in hiding behavior between infected and uninfected males in the field. For activity, only females were affected in the field. Infected females had lower activity levels than uninfected females. Infected females and males appear to experience different physiological effects of infection, with females being more heavily impacted by infection. For body color, *A. dirus* infection was associated with body color in both males and females, causing infected isopods to be lighter than uninfected isopods. This may increase parasite transmission to a visual predator when found within a dark colored habitat. The pigmentation dystrophy observed may be the result of parasitic manipulation or pathological side-effects.

I examined if there was consistency and repeatability in the expression of host focal traits under different contexts, the field and the laboratory. Activity was observed to be consistent and repeatable in most of the groups, whereas the consistency and repeatability of hiding behavior were more variable. Two out of the four groups showed consistency, and none of the groups showed repeatability in their hiding behavior. The mechanism for hiding behavior appears to differ from that of activity. Activity generally remains constant, even under different environmental contexts. This could be useful for future studies involving activity in individual *C. intermedius*.

Consistency and repeatability are related but not synonymous terms that are used to describe behavior (Cummings and Mollaghan 2006). There were discrepancies between the two measures of consistency and repeatability; however, these discrepancies may result from the

calculation of repeatability, which requires parametric data values. The data values used were generally not normally distributed; therefore, non-parametric tests were used. Also, because repeatability is calculated as a ratio, repeatability can appear low, in comparison to consistency, when the variation both within and among individuals is low (Cummings and Mollaghan 2006).

Correlations among Focal Traits and Disruptions in the Correlations

Many parasites that infect intermediate hosts modify numerous aspects of their hosts' phenotypes. Parasitic manipulation of host phenotype may alter multiple traits which could be correlated in terms of increased transmission to the definitive host. Two distinctly altered traits may share a common developmental mechanism, which could result in positive relationships between the levels of each trait and lower costs of manipulation (Cézilly and Perrot-Minnot 2005; Poulin 2010; Thomas et al. 2010). The alteration of additional traits could synergistically increase the efficiency or specificity of the original manipulation by increasing the likelihood of predation of an intermediate host by the definitive host (Médoc et al. 2009). At the same time, the susceptibility to predation by other predators that are unsuitable as definitive hosts for the parasite could decrease (Médoc et al. 2009). Predators that are unsuitable as definitive hosts could include hosts that are unable to sustain a population of parasites within a certain environment or hosts that disrupt the parasite life cycle. Furthermore, the manipulation of hosts by parasites can be pictured as a mosaic of multiple, altered traits. The potential relationships between traits can be explored, which may aid in understanding the evolutionary mechanisms behind a specific manipulation strategy (Benesh et al. 2008). It is possible that in

some cases the correlations between these host traits may be the true targets of manipulations, rather than the traits themselves (Coats et al. 2010; Poulin 2010).

I examined if there were correlations among traits in both infected and uninfected isopods to determine if parasite infection was associated with changes in trait correlations. The results obtained showed that there was a correlation between color score and activity for only females in the field. Dark-colored females were more active than light-colored females. The correlation occurred in the direction opposite to what was predicted. It was predicted that light-colored isopods would be more active than dark-colored isopods. There were no correlations between hiding behavior and either color or activity in the field. There were also no correlations between % color and activity in the field.

I then examined if *A. dirus* infection was associated with a change in the correlations among traits. In the one case where a correlation occurred in uninfected females in the field, there was not a correlation between the traits in the laboratory. Specifically, activity and color, which were correlated in nature, were not correlated in another context. This correlation may not be a meaningful correlation because it was expected that the correlation would occur in a different context, the laboratory, in addition to occurring in the field. Future research is needed to determine the potential significance of the correlations among traits identified in nature and disruptions to these correlations.

There may have been differences in the consistency of the relationships among traits for infected isopods in the field and laboratory for a couple of reasons. The external conditions themselves affected the relationships among traits or measurements of the traits themselves

may have been highly variable. Low repeatability can occur when environmental influences are not sufficiently controlled. Factors, such as temperature or hormonal state, could affect an individual's performance (Boake 1989). Future studies in the laboratory should standardize conditions such as air and water temperature, pH, amount of light, and predatory fish cues. The simulation of natural field conditions is essential if the effects of infection on modified traits are context-dependent.

In contrast to the concerns raised about the correlation identified in nature, this correlation does provide insights into the relationships among traits in *C. intermedius* individuals. In regards to the relationships among traits, the traits may have different underlying mechanisms that are used to modify each trait independently.

Candidate Proximate Mechanisms

In other crustacean hosts, modification of the expression of host traits has also been associated with changes in serotonin (5-HT) and dopamine (DA) (Maynard et al. 1996; Poulin et al. 2003; Rojas and Ojeda 2005; Perrot-Minnot et al. 2014). Serotonergic and dopaminergic systems could also have a role in the modification of the host focal traits and relationships among traits in *C. intermedius* individuals. These systems have been proposed as candidate mechanisms of modification for various parasites across several taxa (Lafferty and Shaw 2013; Perrot-Minnot and Cézilly 2013). If 5-HT or DA was a shared underlying mechanism behind the focal traits, then the focal traits should have been modified in the same direction under

different contexts. Because the focal traits were not modified in the same direction in this study, the focal traits may not have these shared 5-HT and/or DA underlying mechanisms.

Research on this system has shown that 5-HT and DA levels differ between infected and uninfected isopods, with infected isopods containing lower levels of 5-HT and DA than uninfected isopods (Kopp et al. 2016). Effects of infection on neurochemical levels have also been identified in other host-parasite relationships (Shaw et al. 2009). In a similar host-parasite system, an increase in 5-HT in uninfected amphipods mimicked the effects of parasite infection on certain behaviors, with the possible exclusion of refuge use (Perrot-Minnot et al. 2014). In contrast to this study, this other host-parasite system may use 5-HT as a shared underlying mechanism for several of the host traits. Further studies on the role of serotonergic and dopaminergic systems in the modification of the expression of traits in *C. intermedius* individuals could provide insights into the consistency and repeatability of host focal trait expression and relationships among traits.

Correlations between Parasite Characteristics (Intensity and Volume) and Modification of Focal Traits

Parasite characteristics, such as size and intensity, have the potential to impact host modification. Both positive and negative relationships have been identified between the parasite characteristic and the level of modification (Franceschi et al. 2008; Benesh et al. 2009; Dianne et al. 2012; Caddigan et al. 2014). I examined if the modification of host focal traits was correlated with parasite characteristics, such as intensity and volume. There were no

detectable relationships between parasite characteristics and the modification of focal traits in individual *C. intermedius*.

Conclusions

Multiple focal traits in individual *C. intermedius* were associated with *A. dirus* infection. For hiding behavior of individual *C. intermedius*, there was an effect of infection on individuals in the laboratory and an effect of infection only on males in the field. Further studies on effects of *A. dirus* infection associated with hiding behavior of individual *C. intermedius* should increase the sample size of the experiment in order to determine the consistency and repeatability of these observations. For activity, infection affected only females in the field. Infected females displayed lower activity than uninfected females. These changes in female activity appear to be more likely associated with pathological effects of infection than adaptive manipulation because the changes are unlikely to increase transmission, do not occur in males, and pathological effects are generally more pronounced in females than males.

Body color was modified for both males and females, with infected isopods being lighter in color than uninfected isopods. This could increase parasite transmission to a visual predator when found within a dark colored habitat. In different habitats, body color modification may be altered to increase the detection of infected isopods by visual predators (Table 2). Experiments on parasite transmission to predatory final hosts are currently being conducted on the *C. intermedius-A. dirus* relationship (Johnson, J., Sparkes, T. C., unpublished data). The

pigmentation dystrophy observed in this system may have been an adaptive mechanism used by parasites or a pathological side effect of infection.

Further studies on the role of serotonergic and dopaminergic systems in the modification of focal traits in individual *C. intermedius* could also provide insights into the proximate mechanisms underlying the modification of multiple traits and the relationships among multiple traits. For the results obtained here, it appears that different traits may be modulated by different mechanisms because the correlations among traits were not consistent between different contexts.

Limitations of the Approach

For the research presented here, there was a higher than expected level of mortality in the behavioral trials. Mortality could have resulted from high temperatures at the field site, researcher error, or laboratory mortality associated with temperature changes between the field and the laboratory. To address mortality concerns in future studies, shade tents should be used for containers holding isopods during the experiments in the field. Also, more thorough training sessions tailored to student field workers that assist in field and laboratory experiments should be conducted. Lastly, more care in standardizing the temperature of the vials that house the isopods during the transport from the field site to the laboratory should be used.

It should be noted that I used naturally-infected isopods in my trials. Infection was not randomly assigned. Pre-existing differences between infected and uninfected isopods may have

occurred that could have affected the measured phenotypic traits, but the naturally-infected isopod phenotypes resemble those encountered by the definitive hosts in the field (Benesh et al. 2008). Although experimental infections may appear to be the preferred approach, this type of approach can also be problematic because it often results in infection levels that are not found in nature (Benesh et al. 2008). In addition, behavioral traits can exhibit high levels of plasticity. As a result, behaviors expressed in lab-reared animals may be non-representative of behaviors expressed in nature.

Future studies should also incorporate field-based experiments in addition to lab-based experiments to observe the inherent behavior of isopods in nature. Most studies that have previously examined these traits have been conducted in the laboratory. It may be beneficial to observe the typical behavior of isopods in nature before observing their behavior in other contexts, such as in the laboratory. This will establish an appropriate standard of behavior by which to compare behaviors in other contexts. This study was the first to investigate the relationships between infection status in *C. intermedius* individuals and associated effects on host traits, as well as among modified host traits and behaviors in field- and laboratory-based settings. Exploring these relationships further in field- and laboratory-based experiments may continue to add insights into the evolution of both manipulation and transmission strategies in *A. dirus*, as well as in other host-parasite relationships.

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Table 1. Modification of behavioral and physiological traits of isopods infected by cystacanth-stage *Acanthocephalus dirus* (syn. *Acanthocephalus jacksoni*, *Acanthocephalus parksidei*). Traits used in the current study are included in the red box. Traits that have been shown to be modified in *Caecidotea intermedius* in the focal population (Buffalo Creek) are shown with an asterisk. The table presented is a modified version of Table 1 from Korkofigas et al. (2016).

[1]Seidenberg (1973), [2]Oetinger & Nickol (1981), [3]Oetinger & Nickol (1982a), [4]Oetinger & Nickol (1982b), [5]Camp & Huizinga (1979), [6]Camp & Huizinga (1980), [7]Hechtel et al. (1993), [8]Sparkes et al. (2004), [9]Korkofigas (2007), [10]Oetinger (1987), [11]Sparkes et al. (2006), [12]Bierbower & Sparkes (2007), [13]Mormann (2010), [14]Caddigan et al. (2014), [15]Korkofigas et al. (2016), [16]Muzzall & Rabalais (1975a), [17]Muzzall & Rabalais (1975b), [18]Muzzall & Rabalais (1975c).

Modified trait	Effect	Location	Reference(s)
Caecidotea intermedius (IL)			
Body pigmentation*	↓	Lab, Field	1, 2, 3, 4, 5, 6, 7, 8
Refuge use*	↓	Lab	5, 7, 9
Activity	↑	Lab	5
Predator attraction	↑	Lab	7
Mate guarding*	↓	Lab, Field	9, 10, 11, 12
Male mating response*	↓	Lab, Field	11, 12, 13, 14
Egg production*	↓	Field	11
Male energy content*	↑	Field	14, 15
Lirceus lineatus (OH)			
Body pigmentation	↓	Field	2, 4, 16, 17
Refuge use	↓	Lab	18
Egg production	↓	Lab	17
Activity	↑	Lab	18
Lirceus garmani (AK)			
Body pigmentation	↓	Field	2, 4

Table 2. Modification of behavioral and physiological traits of isopods infected by cystacanth-stage *Acanthocephalus* species in various countries.

[1]Seidenberg (1973), [2]Oetinger & Nickol (1981), [3]Oetinger & Nickol (1982a), [4]Oetinger & Nickol (1982b), [5]Camp & Huizinga (1979), [6]Camp & Huizinga (1980), [7]Hechtel et al. (1993), [8]Sparkes et al. (2004), [9]Korkofigas (2007), [10]Muzzall & Rabalais (1975a), [11]Muzzall & Rabalais (1975b), [12]Muzzall & Rabalais (1975c), [13]Benesh et al. (2008), [14]Pilecka-Rapacz (1986), [15]Brattey (1983), [16]Lyndon (1996), [17]Dezfuli et al. (1994)

Parasite	Host-Modified trait	Effect	Location	Reference(s)
Acanthocephalus dirus	*Caecidotea intermedius* (IL, USA)			
	Body pigmentation	↓	Lab, Field	1, 2, 3, 4, 5, 6, 7, 8
	Refuge use	↓	Lab	5, 7, 9
	Activity	↑	Lab	5
Acanthocephalus dirus	*Lirceus lineatus* (OH, USA)			
	Body pigmentation	↓	Field	2, 4, 10, 11
	Refuge use	↓	Lab	12
	Activity	↑	Lab	12
Acanthocephalus dirus	*Lirceus garmani* (AK, USA)			
	Body pigmentation	↓	Field	2, 4
Acanthocephalus lucii	*Asellus aquaticus* (Finland)			
	Body pigmentation	↑	Lab	13
	Refuge use	↓	Lab	13
Acanthocephalus lucii	*Asellus aquaticus* (Poland)			
	Body pigmentation	↑	Lab	14
	Activity	↑	Lab	14
Acanthocephalus lucii	*Asellus aquaticus* (Scotland)			
	Body pigmentation	↑	Lab	15
Acanthocephalus lucii	*Asellus aquaticus* (England)			
	Body pigmentation	↑	Lab	16
Acanthocephalus anguillae	*Asellus aquaticus* (Poland)			
	Body pigmentation	↑	Lab	14
	Activity	↑	Lab	14
Acanthocephalus anguillae	*Asellus aquaticus* (England)			
	Body pigmentation	↑	Lab	16
Acanthocephalus anguillae	*Asellus aquaticus* (Italy)			
	Body pigmentation	↑	Lab	17

Table 3. Traits of the host, infection status of the host, and location of the trials. Shown are median values with upper and lower quartiles in parentheses. a: males. b: females. Open represents the percentage of time that isopods spent in the open. Activity represents the number of grid lines crossed in a five minute period. Color score represents the score assigned based on a color scale (1-9). % color represents the percentage of dark brown color present. ns indicates non-significant differences in probability values.

a. Male

Trait	Location	Infected	Uninfected	p-value
Open	Field	67 (17, 100)	33 (0, 83)	0.04
	Laboratory	100 (100, 100)	100 (42, 100)	0.01
Activity	Field	35 (4, 75)	48 (11, 79)	ns
	Laboratory	30 (7, 66)	38 (18, 67)	ns
Color Score	Field/Laboratory	6 (4, 6)	8 (7, 8)	<0.001
% Color	Field/Laboratory	8 (5, 54)	100 (100, 100)	<0.001

b. Female

Trait	Location	Infected	Uninfected	p-value
Open	Field	50 (13, 83)	33 (4, 67)	ns
	Laboratory	100 (100, 100)	83 (33, 100)	0.001
Activity	Field	27 (5, 63)	63 (28, 83)	0.01
	Laboratory	40 (8, 82)	36 (18, 64)	ns
Color Score	Field/Laboratory	5 (4, 6)	8 (7, 9)	<0.001
% Color	Field/Laboratory	7 (5, 21)	100 (100, 100)	<0.001

Table 4. Estimates of consistency (Spearman correlation coefficients) and repeatability (intra-class correlation coefficients) for each behavior measured in the field and the laboratory. Open represents the percentage of time that isopods spent in the open. Activity represents the number of grid lines crossed in a five minute period. Shown in bold and gray are behaviors that were consistent or repeatable between the two locations.

Trait	Sex	Infection Status	Consistency (r_s)	Repeatability (R)
Activity	Male	Infected	**0.52**	**0.46**
		Uninfected	**0.50**	**0.36**
	Female	Infected	**0.37**	**0.30**
		Uninfected	0.01	-0.05
Open	Male	Infected	**0.48**	0.03
		Uninfected	0.17	0.01
	Female	Infected	-0.16	-0.44
		Uninfected	**0.45**	0.20

Table 5. Partial correlation coefficients between traits for infected and uninfected male and female isopods in the field and laboratory. a: partial correlation coefficients between traits for relationships involving color score. b: partial correlation coefficients between traits for relationships involving % color. Bold font indicates significant relationships between traits. Gray shading indicates significant relationships in the field.

a. Color Score

Trait Combination	Sex	Infection Status	Field	Laboratory	Consistent
Color - Activity	Male	Infected	-0.16	0.17	--
		Uninfected	-0.06	-0.13	--
	Female	Infected	0.17	0.24	--
		Uninfected	**0.32**	0.01	No
Color – Open	Male	Infected	0.24	0.04	--
		Uninfected	-0.07	-0.02	--
	Female	Infected	0.12	0.02	--
		Uninfected	-0.02	0.05	--
Open - Activity	Male	Infected	-0.21	0.18	--
		Uninfected	0.10	0.32	--
	Female	Infected	0.13	0.12	--
		Uninfected	0.05	**0.35**	--

b. % Color

Trait Combination	Sex	Infection Status	Field	Laboratory	Consistent
Color - Activity	Male	Infected	-0.05	0.06	--
		Uninfected	-0.05	-0.16	--
	Female	Infected	0.13	0.12	--
		Uninfected	0.19	-0.23	--
Color - Open	Male	Infected	0.20	-0.08	--
		Uninfected	-0.07	0.03	--
	Female	Infected	0.21	0.03	--
		Uninfected	-0.19	-0.16	--
Open - Activity	Male	Infected	-0.21	0.18	--
		Uninfected	0.10	0.32	--
	Female	Infected	0.13	0.12	--
		Uninfected	0.05	**0.35**	--

Table 6. Spearman correlation coefficients examining the relationships among traits of the host (field measures) and parasite characteristics. a: males. b: females. Body size was measured as body area (mm^2). Color score represents the score assigned based on a color scale (1-9). % color represents the percentage of dark brown color present. Open represents the percentage of time spent in the open. Activity represents the number of grid lines crossed. Bold font and gray shading indicate significant relationships between parasite characteristic and host trait.

a. Male

Trait	Intensity	Average Volume	Total Volume
Body Size (mm^2)	0.19	**0.56**	**0.59**
Color Score	-0.17	0.10	-0.01
% Color	-0.11	0.11	0.04
Open	-0.19	0.15	0.05
Activity	0.02	-0.07	-0.08

b. Female

Trait	Intensity	Average Volume	Total Volume
Body Size (mm^2)	0.10	0.05	0.07
Color Score	-0.19	-0.05	-0.12
% Color	0.02	-0.12	-0.09
Open	-0.13	0.02	-0.02
Activity	-0.21	-0.11	-0.20

Table 7. Host sex, host body size (area in mm^2), and parasite characteristics. Shown are median values with upper and lower quartiles in parentheses. ns indicates non-significant differences in probability values.

	Male	Female	p-value
Host Size (mm^2)	43.88 (26.85, 56.23)	30.73 (23.78, 35.03)	<0.001
Parasite characteristics			
Intensity	1.00 (1.00, 2.00)	1.00 (1.00, 1.00)	ns
Average Volume (mm^3)	1.01 (0.43, 1.71)	0.90 (0.41, 1.40)	ns
Total Volume (mm^3)	1.26 (0.47, 2.33)	1.04 (0.45, 1.66)	ns

Figure 1. Locality and depiction of study site. a: locality of study site at Buffalo Creek in Kildeer, Illinois. b: depiction of study site upstream. c: depiction of study site downstream.

a.


Illinois
Kildeer
Cedar Rapids
Chicago
Fort Wayne
Indianapolis
Springfield
Cincinnati
St. Louis
Louisville
Springfield
© 2005 BestPlaces

b.

c.

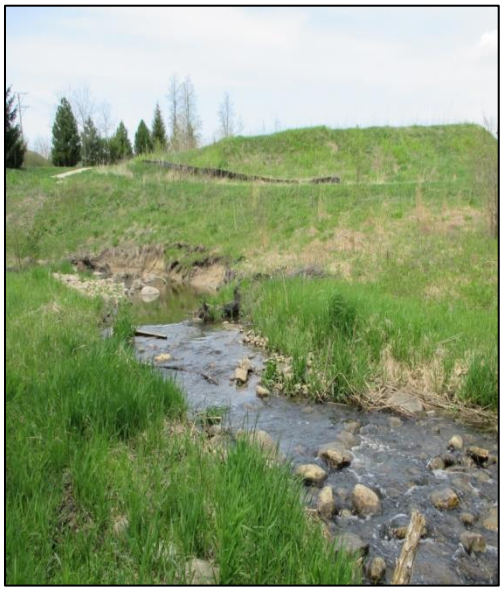
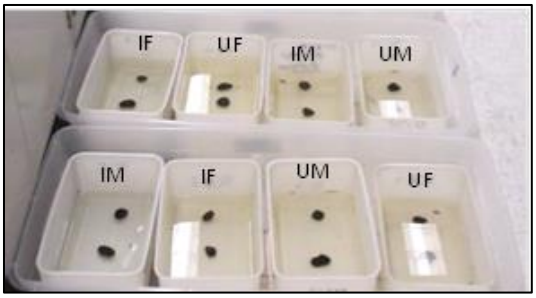


Figure 2. Experimental arenas used for behavioral trials. a: twelve trays housed four experimental arenas each filled with stream water, with one isopod of each group in each arena. The four groups were: IM=infected male, UM=uninfected male, IF=infected female, UF=uninfected female. Forty-eight trials were run simultaneously, which yielded 12 replicates for each group. b: examples of two trays each housing four experimental arenas. c: illustration of the layout for each experimental arena. The dimensions of the different rock sizes are described in the text.

a.


1
2
3
4
5
6
7
8
9
10
11
12

b.


IF
UF
IM
UM
IM
IF
UM
UF

c.

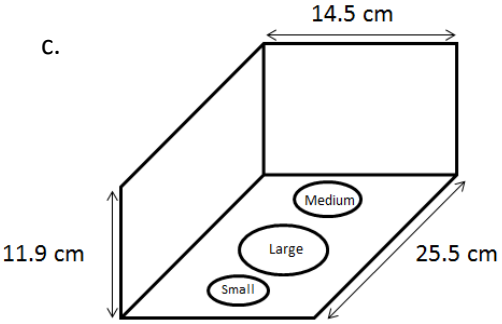
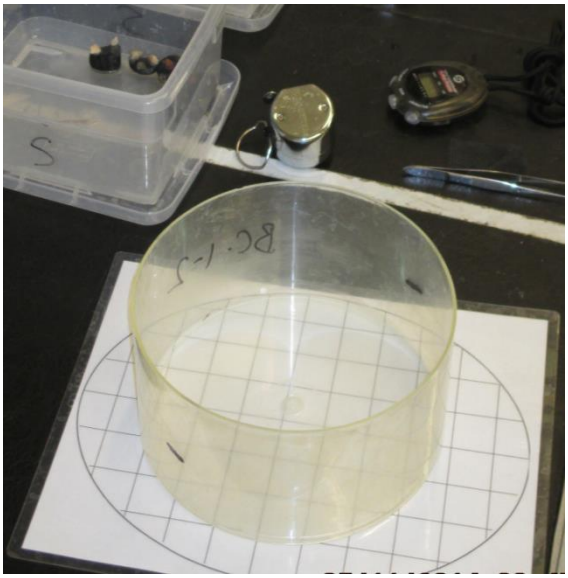

14.5 cm
11.9 cm
25.5 cm
Medium
Large
Small

Figure 3. An example of a round experimental arena (18 cm diameter and 8 cm height) with an example grid sheet (2.5 cm x 2.5 cm grids) placed underneath for the analysis of activity.



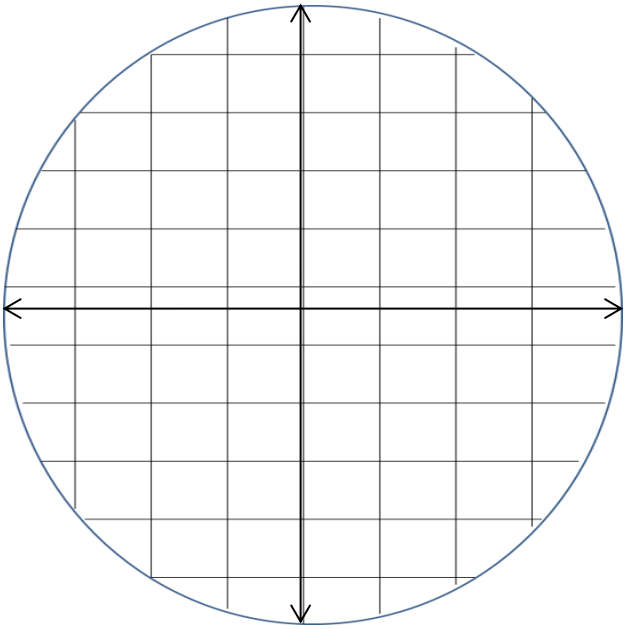
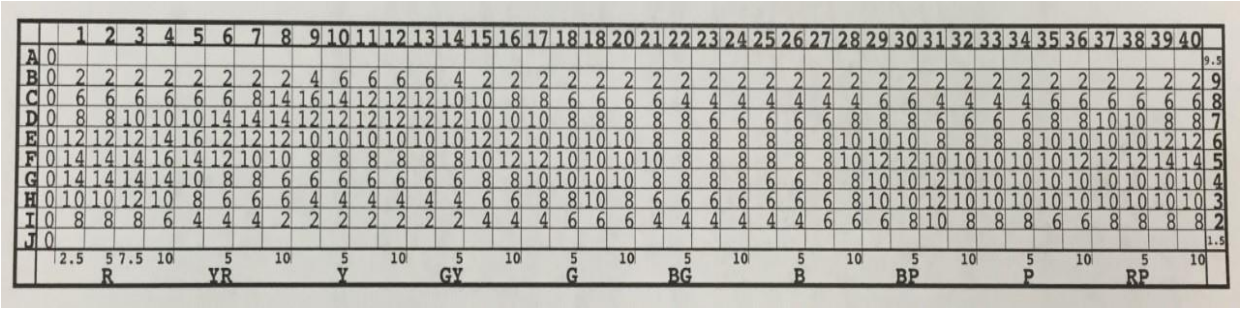


Figure 4. World Color Survey stimulus array used for color analysis (Lenneberg and Roberts 1956). a: columns 5.5 Yellow-Red through 2.5 Yellow were used in the images for color analysis. b: the colors in columns 6-9 correspond to columns 5.5 Yellow-Red through 2.5 Yellow in the first table.

a.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 18 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
A 0
B 0 2 2 2 2 2 2 2 2 4 6 6 6 6 4 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 9
C 0 6 6 6 6 6 6 8 14 16 14 12 12 12 10 10 8 8 6 6 6 6 4 4 4 4 4 4 4 6 6 4 4 4 4 6 6 6 6 6 6 8
D 0 8 8 10 10 10 14 14 14 12 12 12 12 12 12 10 10 10 8 8 8 8 8 6 6 6 6 6 8 8 8 6 6 6 6 8 8 10 10 8 8 7
E 0 12 12 12 14 16 12 12 12 10 10 10 10 10 10 12 12 10 10 10 10 8 8 8 8 8 8 8 10 10 10 8 8 8 8 10 10 10 10 12 12 6
F 0 14 14 14 16 14 12 10 10 8 8 8 8 8 8 10 12 12 10 10 10 10 8 8 8 8 8 8 8 10 12 12 10 10 10 10 10 12 12 12 14 14 5
G 0 14 14 14 14 10 8 8 6 6 6 6 6 6 6 8 8 10 10 10 10 8 8 8 8 6 6 8 8 10 10 12 10 10 10 10 10 10 10 10 10 10 4
H 0 10 10 12 10 8 6 6 6 4 4 4 4 4 4 6 6 8 8 10 8 6 6 6 6 6 6 6 8 10 10 12 10 10 10 10 10 10 10 10 10 3
I 0 8 8 8 6 4 4 4 2 2 2 2 2 2 4 4 4 6 6 6 4 4 4 4 4 4 6 6 6 8 10 8 8 8 6 6 8 8 8 8 2
J 0
2.5 5 7.5 10 5 10 5 10 5 10 5 10 5 10 5 10 5 10 5 10 5 10 5 10
R YR Y GY G BG B BP P RP
9.5
1.5

b.

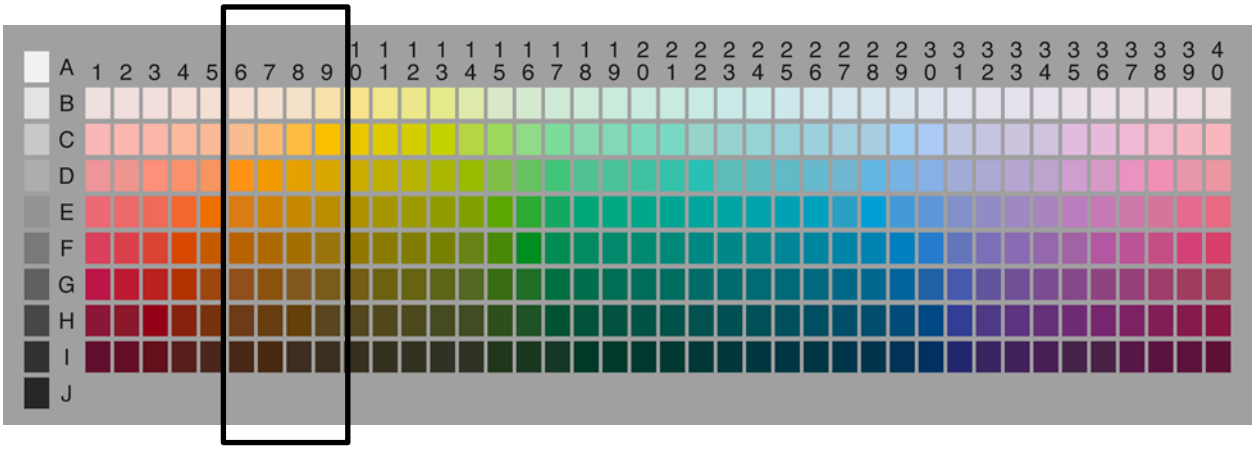

A 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
B
C
D
E
F
G
H
I
J

Figure 5. An example of a captured image of an isopod (ID: BC-2-7) that was used for body color analysis with ImageJ. A one-centimeter section of a ruler was placed with the isopods as a reference for size in the analysis. Color references were used to assign a color score to each isopod (from 1 to 9, labeled above). BC identified Buffalo Creek as the study site, 2 identified the field day, and 7 identified the individual isopod number.

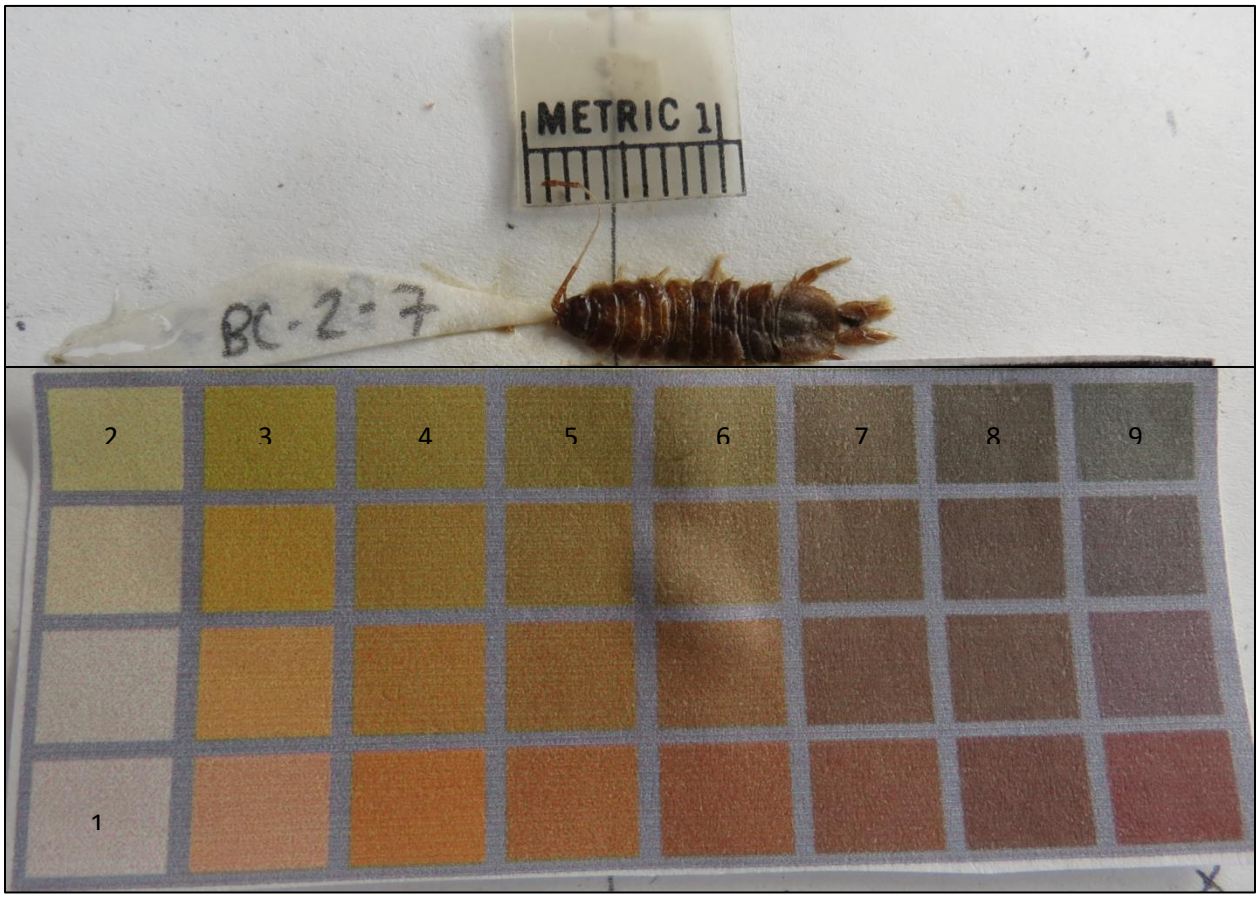

METRIC 1
BC-2-7
2
3
4
5
6
7
8
9
1

Figure 6. Body sizes (in mm^2) of infected and uninfected male and female isopods. *** indicates $p<0.001$. Infected and uninfected isopods differed significantly in body size.

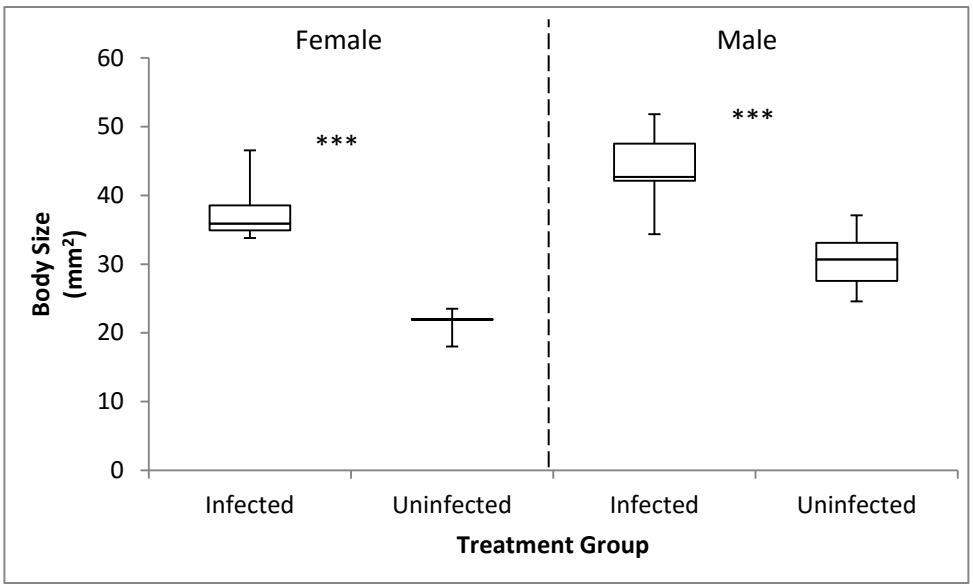
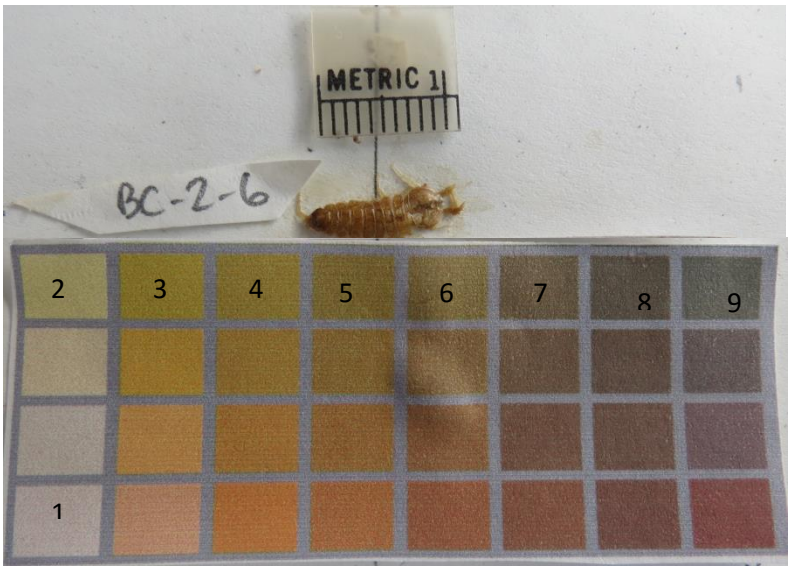

Female
Male
***
***
60
50
40
30
20
10
0
Body Size (mm²)
Infected
Uninfected
Infected
Uninfected
Treatment Group

Figure 7. Captured images of isopods for body color analysis. a: an infected isopod with a color score of 6 (yellow) and a percent color value of 4.41%. b: an uninfected isopod with a color score of 9 (dark brown) and a percent color value of 100%. These are typical scores and values for uninfected isopods.

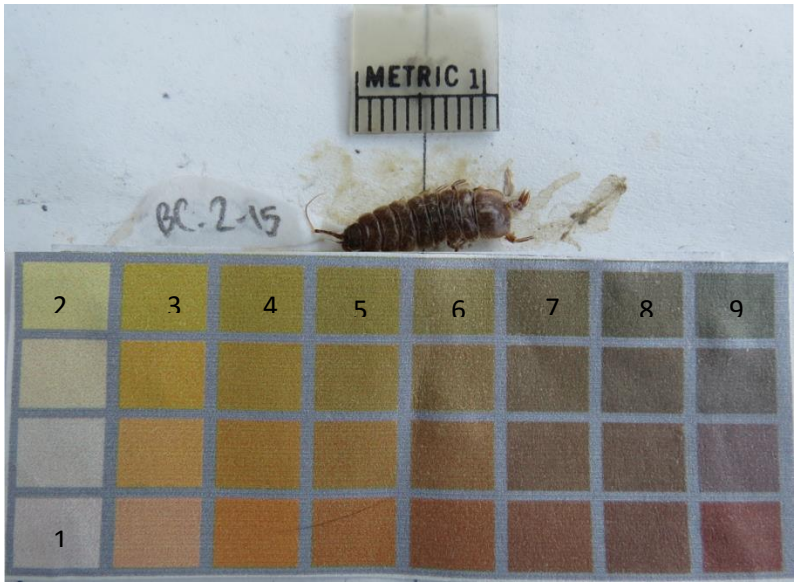
a.



Color Score: 6

% Color: 4.41

b.



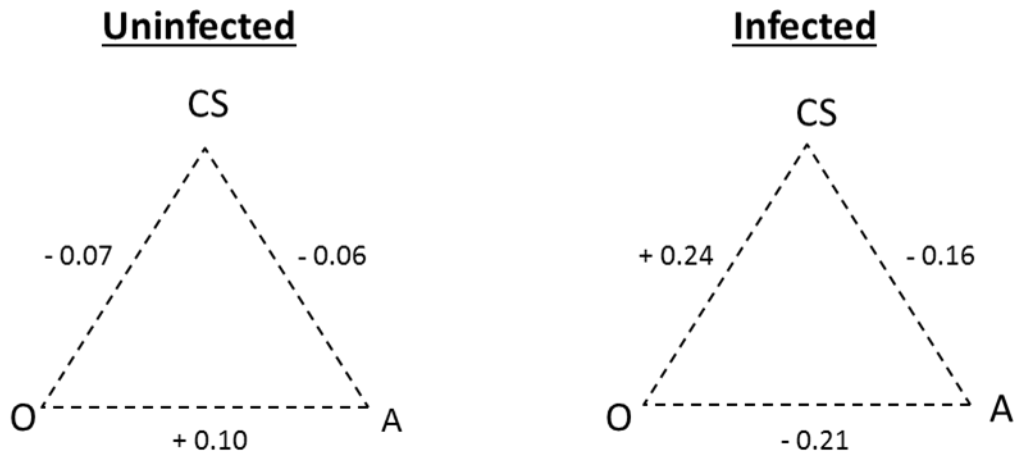
Color Score: 9

% Color: 100

Figure 8. Correlation coefficients between traits for infected and uninfected male and female isopods in the field. a: males. b: females. CS represents the color score, A represents activity (number of grid lines crossed). O represents the percentage of time spent in the open. Solid lines indicate significant relationships between traits. Dashed lines indicate non-significant relationships between traits.

Color Score, Hiding Behavior, and Activity

a) Male



b) Female

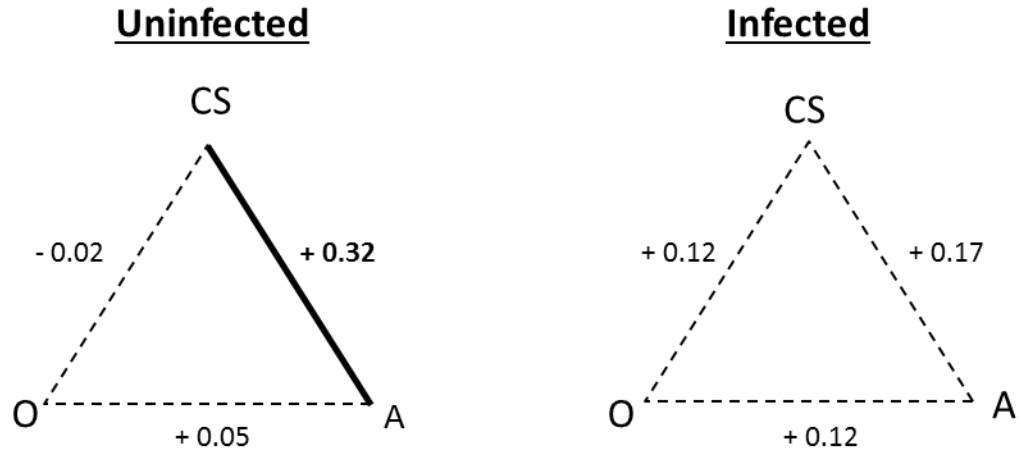
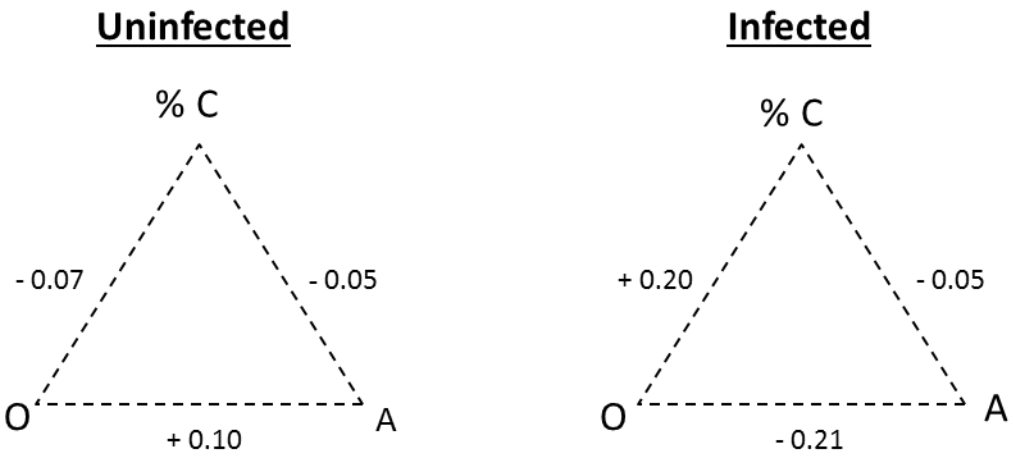


Figure 9. Correlation coefficients between traits for infected and uninfected male and female isopods in the field. a: males. b: females. %C represents the % color. A represents activity (number of grid lines crossed). O represents the percentage of time spent in the open. Dashed lines indicate non-significant relationships between traits.

% Color, Hiding Behavior, and Activity

a) Male



b) Female